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Fibronectin Is the Major Fibroblast Chemoattractant in Rabbit Anti-Glomerular Basement Membrane Disease

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The mechanism for fibroblast recruitment in renal fibrosis due to anti-glomerular basement membrane (anti-GBM) disease is unknown. Since fibroblast recruitment can occur via chemotaxis, assessment of the possible production of fibroblast chemotactic activity by affected renal tissue and its identification could provide important clues. Anti-GBM disease was induced by injection of guinea pig anti-rabbit GBM immunoglobulin G into rabbits previously sensitized to guinea pig immunoglobulin G. On days 4, 7, and 14 after induction, renal tissue was harvested and glomeruli isolated. Overnight serum-free conditioned media from whole cortex and glomeruli were prepared and assayed for fibroblast chemotactic activity. The results show low level activity in both conditioned media from control animals. In contrast, conditioned media from anti-GBM-treated animals at all time points showed significantly elevated fibroblast chemotactic activity peaking on day 4 with subsequent reduction thereafter. The magnitude of increase in cortical conditioned media was significantly higher than that for glomerular conditioned media, suggesting that most of the activity was derived from extraglomerular sources. Gel filtration analysis revealed the activity to be heterogeneous, consisting of at least four major species with estimated molecular weights ranging from 10 to >100 kd. Acidification of conditioned media failed to increase chemotactic activity significantly, whereas protease digestion abolished it. Treatment of conditioned media with antifibronectin inhibited >85% of the chemotactic activity, whereas antibodies to platelet-derived growth factor and transforming growth factor-β did not have a significant effect. These findings taken together suggest that fibronectin-derived peptides represent the predominant fibroblast chemoattractant produced by renal cortex in anti-GBM disease. (Am J Pathol 1996, 148:961–967)

Increased numbers of fibroblasts in fibrotic lesions are responsible for the abnormal deposition of interstitial collagen in many organ systems, including the kidney. Previous studies have shown increased deposition of type I collagen in renal fibrosis accompanying anti-glomerular basement membrane (anti-GBM) disease,[1,2] and subsequently the cell type initially responsible for the increased collagen gene expression is found to be the vascular adventitial fibroblast.[3] The formation of glomerular crescents and subsequent scarring characteristic of this disease suggests that these adventitial fibroblasts may have been recruited to this site from their perivascular adventitial sites by chemoattractants produced at or near the site of glomerular injury. This chemotactic recruitment in conjunction with proliferation of these cells under the influence of certain cytokines and growth factors could result in the active fibrosis seen in this and other models of fibrosis.[4–15] In lung fibrosis the origin of the cells responsible for increased collagen expression is also the perivascular adventitial fibroblasts, although fibroblasts in the peribronchiolar adventitia represent an additional significant component.[15–20] Although these models have shown increased numbers of fibroblasts in fibrotic areas at relatively early stages of disease, the basis for this increase has not been unequivocally established. Increased expression of cytokines and other factors with fibroblast growth-promoting potential strongly

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suggest increased proliferation as a mechanism for this observed increase in the fibroblast population. The contribution and role of chemotactic recruitment in renal fibrosis due to anti-GBM disease have not been previously examined.

Previous studies of renal fibrosis in anti-GBM disease have identified increased expression of certain mediators with known fibroblast chemotactic activity[21–29]; however, their actual determination in terms of this functional activity has not been reported. Furthermore, the identity or identities of the responsible mediator are unknown. In this study, production of fibroblast chemotactic activity was assessed in renal cortical tissue and purified glomeruli isolated therefrom, to see if it was elevated in a rabbit model of anti-GBM disease. Identification of the mediator(s) responsible for any observed activity was then attempted by a combination of physicochemical characterization and immunological techniques.

Materials and Methods

Induction of Anti-GBM Disease

The disease was induced in New Zealand White rabbits as previously described.[8] Briefly, rabbits were sensitized by subcutaneous injection of guinea pig immunoglobulin G (IgG) in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) on day −5. On day 0 the animals were treated with an intravenous injection of guinea pig anti-GBM IgG. Control animals received no injections. On days 0, 4, 7, and 14, the animals were sacrificed, and the kidneys were perfused via the renal artery with phosphate-buffered saline (PBS) followed by iron oxide suspension. The kidneys were then removed, and after removal of the capsule, samples of renal cortex were obtained for preparation of conditioned media and isolation of glomeruli for preparation of glomerular conditioned media.

Isolation of Glomeruli

This was undertaken as previously described.[8–10] Briefly, the iron oxide-containing renal cortical samples were homogenized and passed through a 100 μm nylon screen. The filtrate was then placed on a magnet to isolate the iron-containing glomeruli. This procedure resulted in glomerular preparations, which were >90% pure with minimal contamination by tubules.

Preparation of Conditioned Media

Samples of renal cortex were weighed and then minced to approximately 1 mm^3 pieces, rinsed and then suspended in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mg/ml of bovine serum albumin (BSA) at a concentration of 20 mg wet weight/ml.[8,10] For preparation of glomerular-conditioned media, 1000 glomeruli were suspended in 1 ml of the same media. After 24 hours of incubation at 37°C in a CO_2 incubator, the respective conditioned media were harvested, immediately treated with 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and centrifuged to remove particulates and cells. These conditioned media were then stored frozen at −20°C.

Fibroblast Isolation

Fibroblasts for chemotactic assays were obtained from normal rabbit lungs as previously reported using rat lungs.[18] Briefly, lungs were excised, minced, and incubated in sterile PBS containing 0.5% trypsin (Difco, Detroit, MI) and 0.1% collagenase (Sigma Chemical Co.) with constant stirring for 30 minutes at 37°C. After neutralization of proteolytic activity by addition of fetal bovine serum (FBS), the resulting cell suspension was filtered through sterile gauze and harvested by centrifugation. The pelleted cells were then resuspended in DMEM supplemented with 10% FBS and allowed to become confluent. The media were replaced every 3 days and when confluent the cells were passaged using 0.02% trypsin. Cells from the third passage onward were used in the chemotactic assays. Rabbit renal fibroblasts obtained using similar procedures behave similarly in chemotactic assays (data not shown), but in view of the lower cellular yields relative to rabbit lungs, the study was undertaken using the lung cells.

Chemotactic Assay

Fibroblast chemotactic activity was measured using modified blind-well Boyden microchemotactic chambers as previously described for monocytes.[7,11,30,31] Nucleopore polyvinylpyrrolidone-free membrane filters with 8 μm pore size (NeuroProbe, Cabin John, MD) were precoated with 5 μg/ml gelatin before use.[32] Confluent fibroblasts at between the third and tenth passages were used for these assays. The cells were harvested by brief exposure to 0.05% trypsin and 0.5 mmol/L EDTA in PBS at 5°C. After addition of 10% FBS, the cells were centrifuged, washed once, and counted. The cells were then resuspended in serum-free DMEM containing 2 mg/ml BSA at a concentration of 5×10^5 cells/ml. Test and control samples (28 μl) at the appropriate dilution were placed in the bottom wells of a 48-well microchamber (NeuroProbe), and the prepared filter placed between the lower and upper chamber. Fifty μl of the fibroblast suspension were then placed in the upper well. All substances were assayed in triplicate. After incubating for 3 hours at 37°C in a CO_2 incubator, the chambers were disassembled and the filters removed, fixed in methanol, and stained with 2% toluidine blue.[7,11] After drying, the filters were fixed on glass slides, cover slipped, and quantitated for cells which had migrated to the underside of the filters by microscopic examination. Results were expressed as the average number of cells in 10 randomly chosen noncontiguous high power fields (×400). Where indicated to correct for day-to-day variability, in order to allow comparisons of results from different days, chemotactic activity was expressed as standardized units. Each unit of activity was defined as 1% of the activity in the positive control (100 μg/ml fibronectin) after subtraction of the negative control (media only). Typically <10 cells per high power field (hpf) could be counted in negative control filters, whereas the positive controls were >90 cells/hpf. To confirm chemotactic *versus* chemokinetic activity, checkerboard analysis was undertaken as previously described.[7,11,30–32]

Where indicated, selected samples of conditioned media or other test substances were pretreated with antibodies to fibronectin, platelet-derived growth factor (PDGF), or transforming growth factor-β (TGF-β) before assay. Rabbit fibronectin and monoclonal antibody to rabbit fibronectin were prepared as previously described.[21] Human PDGF was obtained from Collaborative Research (Boston, MA). Anti-porcine TGF-β1 (which also recognizes TGF-β2) and anti-human PDGF were purchased from R&D Systems (St. Paul, MN). These antibodies were shown to cross-react with their respective rabbit antigens in previous studies.[8,33] All antibody treatments were undertaken at a final concentration of 100 μg/ml for 30 minutes before assay. Respective type and class-matched nonimmune Ig controls were used to demonstrate specificity of the antibody.

Gel Filtration Analysis

To estimate molecular weight cortical conditioned media, samples were concentrated 10-fold and injected to a precalibrated gel filtration column (TSK-SW3000, Varian Instruments, Palo Alto, CA) connected to a high performance liquid chromatograph (model 5060, Varian Instruments) and eluted isocratically using PBS as previously described.[34–37] One ml samples were collected and then analyzed for fibroblast chemotactic activity as described above.

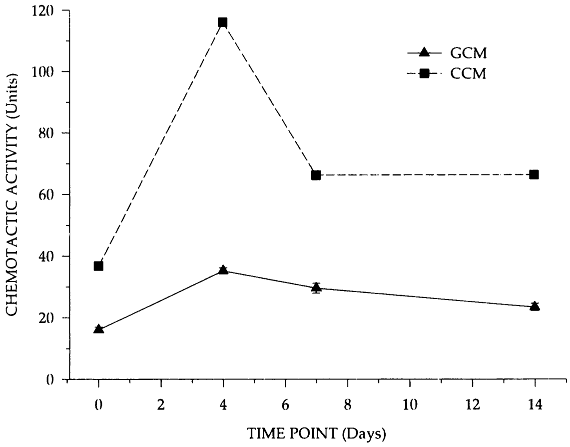

Figure 1. *Fibroblast chemotactic activity of renal conditioned media. At the indicated time points after induction of glomerular injury, renal cortical tissue were harvested. Conditioned media from cortical (CCM) or glomerular preparations (GCM) were assayed for fibroblast chemotactic activity in modified blind well Boyden chambers. Data were expressed as units of activity, with one unit signifying 1% the net activity of 100 μg/ml fibronectin used as the positive control. Means ± SE are shown, with N = 6.*

Statistical Analysis

Differences between mean values were tested for statistical significance using analysis of variance and Scheffe[7,11]

Results

Whole renal cortical samples and isolated glomeruli from control and anti-GBM-treated rabbits were examined for production of fibroblast chemotactic activity. Overnight conditioned media from preparations of control animals revealed measurable activity above that of buffer controls, with activity being roughly doubled in cortical *versus* glomerular conditioned media (Figure 1). Samples from anti-GBM-treated animals at all time points examined contained significantly elevated chemotactic activity (Figure 1). As with the control samples, whole cortical conditioned media contained significantly higher activity than the corresponding glomerular conditioned media. In cortical samples, the elevation in chemotactic activity was maximal at day 4 with more than a threefold increase, which subsequently decreased to nearly a twofold increase over control samples. In glomerular samples, a similar kinetics of increase in chemotactic activity was also observed,

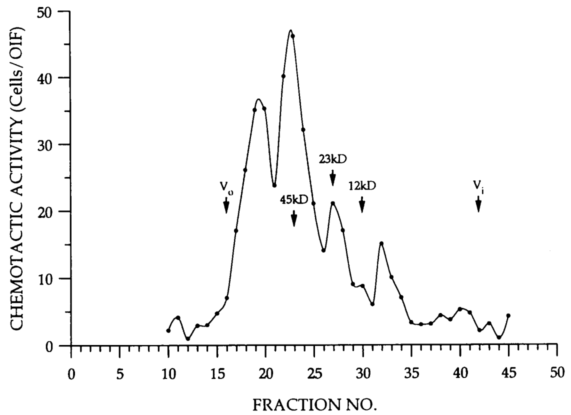

Figure 2. *Gel filtration analysis of day 4 cortical conditioned media. Day 4 cortical conditioned media were concentrated and injected onto high performance liquid chromatography gel filtration column. One ml fractions were collected and assayed for fibroblast chemotactic activity, expressed as the number of migrated cells per oil immersion field (OIF). V_0 and V_i represent the void volume and the retention time for phenol red, respectively. The column was calibrated with the indicated molecular weight standards.*

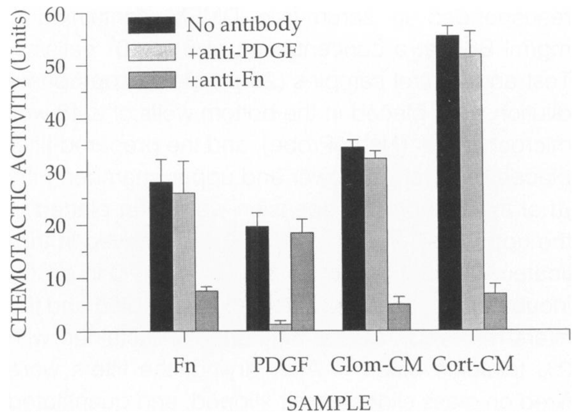

Figure 3. *Effects of antibodies to PDGF and fibronectin on chemotactic activity. Fibroblast chemotactic activity of the indicated substances (Glom-CM, glomerular conditioned media; Cort-CM, cortical conditioned media) were measured as described in Materials and Methods. Certain samples were pretreated with the indicated antibodies prior to assay. Means ± SE are shown with N = 4, representing conditioned media from four animals.*

albeit with a somewhat lower magnitude of increase, being only slightly more than twofold at the peak on day 4. The chemotactic activities in both cortical and glomerular samples from diseased animals were statistically significantly different from their corresponding control values, including the day 14 glomerular samples where a P-value of 0.004 was obtained. Selected samples from day 4 cortical and glomerular conditioned media were tested and the activity found to be truly chemotactic by checkerboard analysis (data not shown).

In order to identify the factor or factors responsible for this chemotactic activity, day 4 cortical conditioned media were concentrated 10-fold and analyzed by gel filtration using a high performance liquid chromatograph. The elution pattern for fibroblast chemotactic activity showed heterogeneity with at least four major molecular species with estimated approximate molecular weights of 10, 24, 50, and 120 kd (Figure 2). Most of the activity appear to be clustered in the 45 to 200 kd region, with significantly less than half of the activity associated with species <50 kd. Since these heterogeneous molecular weights, especially in the larger species, are not helpful in identifying any specific known mediator, a number of physicochemical characteristics were investigated. The activity in day 4 cortical conditioned media was found to be inactivated by heat (100°C for 5 minutes) and pronase digestion (data not shown). These results confirm the proteinaceous nature of the chemotactic factor(s).

A number of proteins are known to be chemotactic for fibroblasts, and a number of these have been found to be expressed in cortex and glomeruli of kidneys from anti-GBM treated rabbits.[8,21,28,29] Since TGF-β was found to be secreted by these kidneys[8,38] and has been reported to be chemotactic for fibroblasts, the effect of prior acidification of conditioned media on fibroblast chemotactic activity was examined. Acidification is known to activate latent TGF-β,[39–41] but it had no significant effect on the observed chemotactic activity in day 4 cortical or glomerular conditioned media. When the samples were pretreated with neutralizing antibody to TGF-β1 and TGF-β2 prior to assay, there was no significant diminution of the observed activity in the same samples (data not shown). When the same samples were then pretreated with neutralizing antibodies to fibronectin and PDGF, only the former significantly inhibited the activity in both conditioned media (Figure 3). Fibroblasts were confirmed to respond chemotactically to pure fibronectin and PDGF, which was inhibited by their respective antibodies. Inhibition of activities in both day 4 cortical and glomerular conditioned media by the anti-fibronectin antibody was >80%, which was not significantly increased by doubling the dose of antibody. Thus, there is a small amount of activity that appear not to be due to fibronectin.

To confirm that fibronectin is the major chemotactic factor in the conditioned media from diseased cortex, day 4 cortical conditioned media were tested to see whether incubation of target fibroblasts by fibronectin and other substances would reduce the chemoattractant activity of these samples. In this experiment, fibroblasts were incubated in the upper chambers or wells of the microchemotactic chamber setup with increasing concentrations of test substance, which included

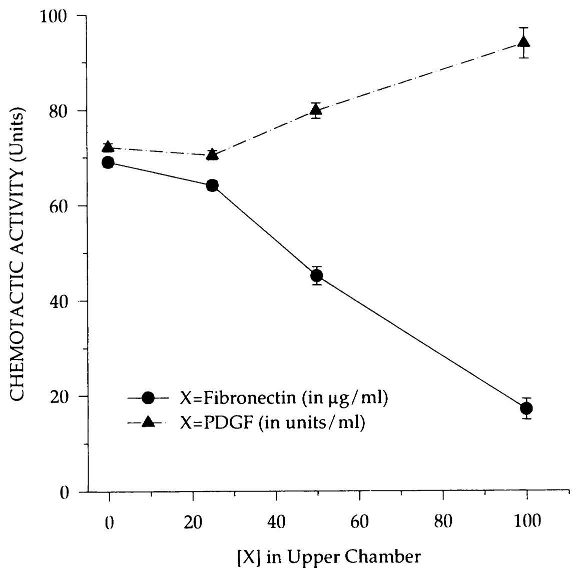

Figure 4. *Desensitizing effects of PDGF and fibronectin. Cells were incubated with either PDGF or fibronectin in the upper chamber and tested for the ability to migrate to day 4 cortical conditioned media placed in the lower chamber. Reduction in activity or desensitization occurred only when the cells were exposed to increasing doses of fibronectin. Means ± SE are shown with N = 4.*

fibronectin, PDGF, tumor necrosis factor-α, and interleukin-1β. These substances were all found to be chemotactic for these fibroblasts (data not shown). A fixed dose of day 4 cortical conditioned media was placed in the lower chamber and the whole assembly incubated as for the standard fibroblast chemotactic assay. Doses of fibronectin <20 μg/ml were found to inhibit or desensitize the chemotactic response of these cells to the day 4 conditioned media in a dose-dependent manner (Figure 4). In contrast, there was no desensitization or inhibition by all the other agents tested (only response to PDGF shown in Figure 4), thus confirming that the bulk of the chemotactic activity in conditioned media was due to fibronectin or fibronectin fragments.

Discussion

Previous studies have demonstrated the perivascular adventitial fibroblast as the major cell type responsible for increased collagen gene expression in kidneys of animals with anti-GBM disease and renal fibrosis.[2,3] The disease eventually progresses to affect the entire glomeruli with evidence of increased numbers of such cells in the fibrotic areas. The results of this study show that significant elevations in secretion of fibroblast chemotactic activity by diseased tissues at similar time points were observed. The nature of the activity appeared heterogeneous and proteinaceous in nature. Despite previous demonstration of elevated production of TGF-β activity by these tissues,[38] most of the activity in these conditioned media was not significantly inhibited by pretreatment with neutralizing antibody to TGF-β1 and TGF-β2. Furthermore, prior acidification, which is known to activate latent TGF-β, failed to enhance the chemotactic activity of the samples. Target fibroblasts were not desensitized to the chemotactic activity of conditioned media by treatment with TGF-β1. Similar negative results were obtained with antibodies to PDGF or desensitization experiments with PDGF, tumor necrosis factor-α, or interleukin 1β, all of which were chemotactic for these cells. In contrast, the activity was inhibited by >80% when the conditioned media were treated with antifibronectin antibodies, while the fibroblasts were desensitized upon treatment with fibronectin. With the heterogeneity in molecular species by gel filtration analysis, the antibody and desensitization studies taken together suggest that fibronectin fragments were responsible for the observed chemotactic activity secreted by diseased cortical and glomerular samples.

The chemotactic activity of fibronectin is well documented and appears to be the dominant species secreted by alveolar macrophages in pulmonary fibrosis as well.[42–52] The activity secreted by alveolar macrophages appears to be associated with the gelatin-binding domain of the fibronectin molecule with molecular weights that are within the range observed in this study. The source of the fibronectin in this model of anti-GBM disease is undetermined, although similar cell types are expected to play important roles in the kidney. The finding that little if any of the chemotactic activity was due to TGF-β is somewhat surprising, given the previous demonstration of TGF-β activity in conditioned media of similar samples studied previously.[8] Despite this finding, however, the role of TGF-β in fibroblast chemotactic recruitment cannot be entirely ruled out because the dose range of this cytokine present in the conditioned media may be outside that for its chemotactic activity. Nevertheless, within the dose range tested, the contribution of TGF-β can be excluded in this *in vitro* study. The situation may be somewhat different *in vivo*, where different concentrations of this and other chemotactic cytokines may be encountered.

The finding that fibronectin fragments may be responsible for the chemotactic recruitment of fibroblasts in anti-GBM disease is consistent with the observed increase in fibronectin gene expression by renal tissue in this model,[21] and the recent demonstration that embryonic fibronectin isoforms are synthesized in glomerular crescents in a similar anti-GBM model in rats.[53] This correlation suggests the potential *in vivo* relevance of these *in vitro* findings.

Thus, recruitment of the collagen-producing perivascular adventitial cells to the periglomerular and glomerular regions by fibronectin may lay the groundwork for progression of the fibrotic process. The recruited cells could then be stimulated by other mediators and cytokines produced by the inflammatory and other cells constituting the crescents, resulting in their proliferation and stimulation of extracellular matrix gene expression and deposition.

Acknowledgments

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